To: McQueen, Jacqueline[McQueen.Jacqueline@epa.gov]
From: Nancy A. Nord
Sent: Thur 3/3/2016 8:18:17 PM
Subject: RE: test
Brown Univ Study.docx

Jackie, When we talked last week, I briefly described research that is being undertaken up at Brown University on the issue of the carcinogenic properties of recycled rubber. A summary of that study is attached. The study will look at samples of virgin recycled rubber, rubber from five-year-old fields and from field soil and the cell mutagenic effects induced by those samples. It is anticipated that the study will take approximately 12 weeks to conduct. The scientists at Brown are more than willing to talk with you directly about the details of this study.

Please note that this study is limited to a cell-based assessment of the molecular carcinogenicity of recycled rubber. Follow-up animal studies would be helpful to confirm the findings of the phase 1 initial study. Those animal studies are outside the scope of what is being initially proposed here.

My clients, the Recycled Rubber Council and the Safe Fields Alliance, will describe this study in their comments to the federal register notice. However, as you and I discussed, we wanted to bring this to your attention now to make sure that you or your colleagues are aware of it as you plan your research efforts. I would greatly appreciate your getting this summary to the relevant staff in your office and if there are questions or concerns, please let me know that as we want to make sure that any additional scientific work is responsive to your needs here.

I look forward to hearing from you.

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-----Original Message-----
From: McQueen, Jacqueline [mailto:McQueen.Jacqueline@epa.gov]
Sent: Wednesday, February 24, 2016 6:54 AM
To: Nancy A. Nord
Subject: RE: test

Thanks, I can't say right now what kind of input, if any, we might have but you are welcome to send me something so we can see what you are proposing and go from there.

Jackie

Jacqueline McQueen
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-----Original Message-----

From: Nancy A. Nord [mailto:nnord@ofwlaw.com]
Sent: Tuesday, February 23, 2016 7:16 PM
To: McQueen, Jacqueline <McQueen.Jacqueline@epa.gov>
Subject: RE: test

Enjoyed our conversation this afternoon re the crumb rubber issue. I will talk to my clients about how best to get you the research they are doing and get back to you.

Nancy A. Nord, Esq.

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From: McQueen, Jacqueline [McQueen.Jacqueline@epa.gov]
Sent: Tuesday, February 23, 2016 2:34 PM
To: Nancy A. Nord
Subject: test

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Evaluation of the potential carcinogenic and tumor-promoting effects of crumb rubber

Introduction:

Recent public questions as to the safety of crumb rubber (CR) used in playing fields can be answered by modern scientific analysis. In this proposal, the potential carcinogenicity of 1) virgin CR, and 2) Field soil will be assessed using the latest, most authoritative methods. Establishing the safety of virgin CR is the primary goal. Establishing the safety of environmentally aged CR is also important, because increased cancer rates may be caused by chemical breakdown or by deposited environmental pollutants and will be considered after the initial analyses.

Rapid *in vitro* cell transformation assays will be used to assess the molecular carcinogenicity of CR in Phase 1 studies, which will establish an initial baseline of safety. Subsequently, should results confirm that virgin CR is not carcinogenic in cell culture, a more expanded study will be conducted followed by Phase 2 studies using a rapid *in vivo transgenic* mouse model to confirm or refute these data. Whole animal studies allow for the impact of potential carcinogens on the varied cell types that compose the body in a complex physiological setting. *In vivo* testing in rodents remains the widely accepted gold standard by regulatory agencies in the US and Europe, and will be used to confirm and reinforce the Phase 1 data, making the conclusion unassailable by the best of available technology.

Genetic toxicity testing is routinely performed to identify potential genotoxic carcinogens and germ cell mutagens. The purpose of the *in vitro* mammalian cell gene mutation test is to detect gene mutations induced by chemical substances. The cell lines used in these tests measure forward mutations in reporter genes, specifically the endogenous hypoxanthine-guanine phosphoribosyl transferase gene (*HPRT* in human cells). The HPRT test will detect mutational events, if any, such as base pair substitutions, frameshifts, small deletions and insertions (1-3).

The *in vitro* micronucleus test is a genotoxicity test for the detection of micronuclei (MN) in the cytoplasm of interphase cells. Micronuclei may originate from acentric chromosome fragments (*i.e.* lacking a centromere), or whole chromosomes that are unable to migrate to the poles during the anaphase stage of cell division. Therefore the MN test is an *in vitro* method that provides a comprehensive basis for investigating chromosome damaging potential *in vitro* that can be transmitted to daughter cells (4,5).

These models (HPRT and MN), for the phase 1 studies, will utilize *in vitro* genotoxicity tests recommended by regulatory agencies to detect genotoxic carcinogens, if any, from bioaccessible CR. Because each *in vitro* genotoxicity test has its own advantages and disadvantages, a set of such tests is needed to obtain meaningful preliminary results.

Because compounds can potentially cooperate to cause cancer, we propose to use bioaccessible extracts of CR for carcinogenicity testing. Bioaccessibility simulates the interaction of CR with simulated human biological fluids from typical routes of exposure, such lung, sweat and digestive juices (6). In a previous study, levels of potentially toxic substances were found to pose low risk to human health (6). The complex mix of

bioaccessible compounds extracted will be tested for carcinogenicity. This represents an important improvement over prior studies.

Researchers have noted that the *in vitro HPRT* and *in vitro* MN assays in mammalian cell lines were very sensitive and frequently used to confirm the genotoxicity of a broad range of chemicals and substances (7-9). These studies are performed in the presence and absence of S9 liver homogenate to activate metabolism of the pro-compound/carcinogen by the cytochrome p450 system, if necessary.

Phase 1 Studies, Part A: Carcinogenicity of Virgin Crumb Rubber

The goal of these initial Phase 1, Part A studies is to ascertain whether virgin crumb rubber is carcinogenic in cell-based assays, which will provide initial evidence for the presence or absence of carcinogenicity. Subsequent studies will be needed to test whether crumb rubber exposed to the environment is carcinogenic, since ultimately it is this material, which comes in contact with people. Cell-based assays are not considered conclusive, but are used to establish preliminary safety. Therefore, animal studies would strengthen any cell-based data.

The proposed studies will be performed using a double-blind procedure to ensure experimental bias is eliminated: coded samples will be evaluated at an external facility that does not know the true identity of the samples.

For phase 1 studies, we will employ a cell culture experimental approach based on Organization for Economic Co-operation and Development (OECD) test guidelines to assess the CR extracts in the following assays:

1. Hypoxanthine phosphorybosyl transferase (HPRT) Assay (OECD TG 476. 7/28/2015). The HPRT gene is on the X chromosome of mammalian cells, and it is used as a model gene to investigate gene mutations in mammalian cell lines. The assay can detect a wide range of chemicals capable of causing DNA damage that leads to gene mutation (1-3). Mutant cells deficient in Hprt enzyme activity in the HPRT are resistant to the cytostatic effects of the purine analogue 6-thioguanine (TG). The Hprt (in the HPRT test) proficient cells are sensitive to TG, which causes the inhibition of cellular metabolism and halts further cell division. Thus, mutant cells are able to proliferate in the presence of TG, whereas normal cells, which contain the Hprt (in the HPRT test) enzyme, are not. Cytotoxicity is determined by relative survival (RS), i.e., cloning efficiency measured immediately after treatment and adjusted for any cell loss during treatment as compared to the negative control. The treated cultures are maintained in growth medium for a sufficient period of time, characteristic of each cell type, to allow near-optimal phenotypic expression of induced mutations (typically a minimum of 7-9 days). Following phenotypic expression, mutant frequency is determined by seeding known numbers of cells in medium containing the selective agent to detect mutant colonies, and in medium without selective agent to determine the cloning efficiency (viability). After a suitable incubation time, colonies are counted. Mutant frequency is calculated based on the number of mutant colonies

corrected by the cloning efficiency at the time of mutant selection. Cell lines are checked routinely for the stability of the modal chromosome number and the absence of mycoplasma contamination. The normal cell cycle time used in the testing laboratory will be established and be consistent with the published cell characteristics. The spontaneous mutant frequency in the master cell stock will also be checked, and the stock will not be used if the mutant frequency is not acceptable. Prior to use in this test, the cultures will be cleansed of pre- existing mutant cells, e.g.by culturing in HAT medium for HPRT test. A HPRT assay kit is available from PRECICE HPRT Assay Kit, Ref. #K0709- 01-2. [abridged from OECD TG 476, 7/28/2015]

2. *In Vitro* Micronucleus assay (OECD TG 487, 9/26/2014). A micronucleus (MN) test is a test used in toxicological screening for potential genotoxic compounds. The assay is now recognized as one of the most successful and reliable assays for genotoxic carcinogens, i.e., carcinogens that act by causing genetic damage and is the OECD guideline for the testing of chemicals (7). One of the most important considerations in the performance of the MN test is ensuring that the cells being scored have completed mitosis during the treatment or the post-treatment incubation period, if one is used. During or after exposure to CR, the cells are grown for a period sufficient to allow chromosome damage or other effects on cell cycle/cell division to lead to the formation of MN in interphase cells. For induction of aneuploidy, CR will ordinarily be present during mitosis. Harvested and stained interphase cells are analysed for the presence of MN. Ideally, MN will only be scored in those cells that have completed mitosis during exposure to CR or during the post-treatment period, if one is used. In cultures that have been treated with a cytokinesis blocker, this is easily achieved by scoring only binucleate cells. In the absence of a cytokinesis blocker, it is important to demonstrate that the cells analysed are likely to have undergone cell division, based on an increase in the cell population, during or after exposure to the test chemical. For all protocols, it is important to demonstrate that cell proliferation has occurred in both the control and treated cultures, and the extent of test chemical-induced cytotoxicity or cytostasis will be assessed in all of the cultures that are scored for micronuclei. Cytochalasin B (CytoB) is the agent that has been most widely used to block cytokinesis because it inhibits actin assembly, and thus prevents separation of daughter cells after mitosis, leading to the formation of binucleate cells. CytoB will not exceed 1% DMSO (v/v); otherwise, untreated controls will be used to ensure that the percentage of organic solvent has no adverse effect. Alternative methods to counting MN is the use of flow cytometry kit. This kit is available from Litron Labs, Rochester, NY and is acceptable for GLP submissions. [abridged from OECD TG 487, 9/26/2014]

The results of these two assays are complementary (8).

Cell models: These models are being used, in part, for the specificity and association with the biological fluids that will be used. All cells are available from the American Type Culture Collection and will be mycoplasma free.

A) V79 lung fibroblast cells (10), acceptable by OECD, will be used with lung fluid for HPRT and MN assays

B) TK6 lymphoblastoid cells acceptable by OECD, will be used with gastric and sweat fluids for the HPRT and MN assays.